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Investigations on hydrogen isotope ratios of endogenous urinary steroids: reference-population-based thresholds and proof-of-concept

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Carbon isotope ratio (CIR) analysis has been routinely and successfully used in sports drug testing for many years to uncover the misuse of endogenous steroids. One limitation of the method is the availability of steroid preparations exhibiting CIRs equal to endogenous steroids. To overcome this problem, hydrogen isotope ratios (HIR) of endogenous urinary steroids were investigated as a potential complement; results obtained from a reference population of 67 individuals are presented herein. An established sample preparation method was modified and improved to enable separate measurements of each analyte of interest where possible. From the fraction of glucuronidated steroids; pregnanediol, 16-androstenol, 11-ketoetiocholanolone, androsterone (A), etiocholanolone (E), dehydroepiandrosterone (D), 5α- and 5β-androstanediol, testosterone and epitestosterone were included. In addition, sulfate conjugates of A, E, D, epiandrosterone and 17α- and 17β-androstenediol were considered and analyzed after acidic solvolysis. The obtained results enabled the calculation of the first reference-population-based thresholds for HIR of urinary steroids that can readily be applied to routine doping control samples. Proof-of-concept was accomplished by investigating urine specimens collected after a single oral application of testosterone-undecanoate. The HIR of most testosterone metabolites were found to be significantly influenced by the exogenous steroid beyond the established threshold values. Additionally, one regular doping control sample with an extraordinary testosterone/epitestosterone ratio of 100 without suspicious CIR was subjected to the complementary methodology of HIR analysis. The HIR data eventually provided evidence for the exogenous origin of urinary testosterone metabolites. Despite further investigations on HIR being advisable to corroborate the presented reference-population-based thresholds, the developed method proved to be a new tool supporting modern sports drug testing procedures. Copyright © 2012 John Wiley & Sons, Ltd.

Keywords: hydrogen isotope ratio; reference population; excretion study; doping control; GC/TC/IRMS

Introduction

Within the last few years, the number of investigations on carbon isotope ratios (CIRs) of endogenous urinary steroids has increased rapidly. The number of steroids under investigation has grown constantly[1–11] and the number of investigated samples has increased, especially with regard to the generation of reference populations.[6,8,12–16] The existing data demonstrated a large intra- and inter-individual variability in CIRs for different steroids. While the intra-individual variation is perfectly reflected by population-based threshold values, the inter-individual variation limits the applicability of the methodology, i.e. the differences found for steroids within the individuals are small but significant and regarding the Δ values reference-based thresholds will cover these differences. The spread of absolute values might become problematic as individuals from northern Europe in particular showed strongly depleted CIRs for endogenous steroids.[16] This, in combination with recent investigations on CIRs of seized testosterone preparations showing a considerable amount of CIR-enriched pharmaceuticals,[17,18] highlights the possibility of illicit testosterone applications that might remain undetected by common sports drug testing procedures focusing on carbon isotopes only.

For doping control laboratories, one option to deal with this new challenge is to exploit the information on target analytes provided by hydrogen isotope ratios (HIRs). First investigations on HIRs of plant sterols date back to 1999[19] and first measurements of urinary steroids followed in 2004.[20] Four years later, a comprehensive method for the determination of HIRs of endogenous urinary steroids was developed, validated, and published.[21] This approach was improved and expanded regarding the number of measurable steroids and used to establish a reference population and allow for subsequent calculations of reference-population-based threshold values.

HIR are expressed as δ^2H values against the international standard Vienna Standard Mean Ocean Water (VSMOW) based on Equation (1):

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$$\delta^2 H_{VSMOW} = \frac{\left({}^2H/{}^1H\right)_{sample}}{\left({}^2H/{}^1H\right)_{std}} - 1 \quad (1)$$

where $^2H/^1H$ refers to the isotopic composition of sample or standard (std).[22]

As it can be expected that the absolute HIRs of urinary steroids will cover at least a range as broad as CIRs, differences between steroids originating from discrete biological pathways will be detectable by HIR analysis, too. These differences between endogenous reference compounds (ERC) – such as pregnanediol (PD) and target compounds (TC) like testosterone (TESTO) – are expressed as Δ-values based on Equation (2):

$$\Delta[‰] = \delta^2 H_{ERC} - \delta^2 H_{TC} \quad (2)$$

As found for CIRs, Δ-values established by means of reference-population-based studies will cover both the intra- and inter-individual variations as well as measurement uncertainties. Therefore, the thresholds presented herein for different Δ-values can readily be applied to routine doping control samples.

The steroids implemented in this complementary approach were as follows: 3α-Hydroxy-5β-androstane-11,17-dione (11KETO), 5β-pregnane-3α,20α-diol (PD), 5α-androst-16-en-3α-ol (16EN), 17β-hydroxy-androst-4-en-3-one (TESTO), 17α-hydroxy-androst-4-en-3-one (EPIT), 3β-hydroxy-androst-5-en-17-one (DHEA), 5α-androstane-3α,17β-diol (5aDIOL), 5β-androstane-3α,17β-diol (5bDIOL), 3α-hydroxy-5α-androstan-17-one (ANDRO) and 3α-hydroxy-5β-androstan-17-one (ETIO) excreted into urine as glucuronide conjugates. Moreover steroid sulfates of ETIO, ANDRO, DHEA and 3β-hydroxy-5α-androstan-17-one (EPIA), androst-5-ene-3β,17β-diol (5EN17b) and androst-5-ene-3β,17α-diol (5EN17a) were implemented.

In order to test both the method and the established new threshold values for their applicability, an excretion study with TESTO-undecanoate administered orally was performed. Additionally, one doping control sample with a strongly elevated TESTO/EPIT ratio without suspicious CIR values was investigated proving the complementary nature of the derived information as the HIRs were significantly enriched in this specimen.

Experimental

Chemicals and steroids

Bakerbond™ SPE Octadecyl columns (6 ml, 500 mg) were purchased from J.T. Baker (Deventer, the Netherlands). Pyridine and acetic anhydride were from Sigma-Aldrich (Buchs, Switzerland) and β-glucuronidase from *Escherichia coli* from Roche Diagnostics GmbH (Mannheim, Germany). TBME was obtained from Acros (Geel, Belgium), methanol from Merck (Darmstadt, Germany) and acetonitrile from Biosolve (Valkensward, the Netherlands). All solvents and reagents were of analytical grade.

Steroid reference material 5aDIOL, 5bDIOL, 16EN, 11KETO, 5EN17b, 5EN17a, EPIA and 3β-hydroxy-5α-androstane (RSTD) were supplied by Steraloids (Newport, RI, USA). ANDRO, ETIO, PD, DHEA, EPIT, TESTO and TESTO-undecanoate were supplied by Sigma-Aldrich (Steinheim, Germany). Two certified reference materials were purchased from A. Schimmelmann (Indiana University, IN, USA), namely the n-alkane mixture C3 (CAL1) and 5α-androstane batch#2 (CAL2) to calibrate the hydrogen tank gas (Carbagas, Gümlingen, Switzerland).

Reference population

Urine specimens from 38 males and 29 females, aged from 20 to 54 years were collected in the forenoon and stored frozen at -20 °C until analysis. All volunteers were healthy, performed regular exercise up to 20 h/week and were recruited from students and employees of the German Sports University and the Swiss Laboratory for Doping Analysis. All volunteers were asked to fill in a short questionnaire. The study was approved by the local ethics committee of the German Sport University Cologne, and written consent was given by all participants.

Excretion study

One healthy male volunteer (37 years, 82 kg, 180 cm) administered orally 100 mg of TESTO-undecanoate dissolved in ethanol/water (40/60 *v/v*). Prior to the administration study, the $\delta^{13}C$ and δ^2H values of both TESTO-undecanoate and the cleaved TESTO were determined. For the cleavage of the ester the method also applied by Cawley *et al.* was used.[17]

Three negative control urine samples were collected before drug administration and then all urine specimens were collected for the following two days. Subsequently, only the morning and one evening urine were sampled for three days. This protocol resulted in a total number of 21 urine samples. All specimens were stored frozen until preparation.

The ethics committee of the canton Vaud (Protocol 155/11) and Swissmedic (Ref. No. 2011DR3149) approved the study, and the participant gave written consent.

GC-MS measurements

Identification

In order to detect and identify co-elutions and to ensure the absence of any disturbing matrix components in some fractions, it was necessary to scan samples on a GC-MS (gas chromatography-mass spectrometry) system using equivalent chromatographic conditions to the IRMS (isotope ratio mass spectrometry) set-up whilst method development. For this purpose, a GC Agilent 6890 N coupled to a mass selective detector MSD Agilent 5973Network was used (Agilent Technologies, Basel, Switzerland). The GC system was equipped with a J&W Scientific DB-17MS (length 30 m, i.d. 0.25 mm, film thickness 0.25 μm) column from Agilent. The initial oven temperature of 80 °C was maintained for 1 min and increased at 30 °C/min up to 260 °C, then at 3 °C/min to 280 °C and then at 30 °C/min up to 300 °C and kept for 2 min. The injections were performed splitless at 280 °C with 2 μl injection volume. A constant flow of 1.5 ml/min with helium as carrier gas was used. The MSD acquired data in scan mode from *m/z* 40 to 400 and mass spectral data were compared to those from standards.

Quantification

An aliquot of each specimen was prepared according to routine doping control sample preparation procedures to determine the amount of different endogenous steroids.[23] This allowed for determination of urine volume requisite for IRMS.

As the steroid profile determined for each sample only covers glucuronidated steroids, the sulfates had to be quantified separately. A similar approach as the already published one was

chosen,[8] using some simplifications like passing on a sulfated internal standard as within this study only the volume requisite for reconstitution prior to isotope measurements was of interest and not the absolute concentration in urine.

Sample preparation

Analytes have to be efficiently isolated and purified before gas chromatography/thermal conversion/isotope ratio mass spectrometry (GC/TC/IRMS) analysis in order to avoid co-elution of compounds and to keep in readiness the ability to measure differently concentrated urinary steroids in comparable amounts. Both aspects are as necessary for valid HIR determinations as for CIR measurements. Therefore, the already developed extensive sample preparation followed by two-fold high performance liquid chromatography (HPLC) clean-up was further improved.

A detailed description of sample preparation to yield both glucuronidated and sulfated steroids was published elsewhere[8,11,14,21] and will herein only be described in brief: 10–30 ml of urine was applied to a conditioned C18 solid-phase extraction cartridge, washed with 2 ml of water and eluted with three volumes of 1 ml of methanol each. The dried residue was dissolved in 1.5 ml of sodium phosphate buffer and extracted with 4 ml of TBME to separate unconjugated steroids; the aqueous residue was hydrolyzed with β-glucuronidase for 60 min at 50 °C and again extracted with 2 volumes of 4 ml of TBME. The organic layer (containing formerly glucuronidated steroids) was evaporated to dryness, re-dissolved in 2 times 100 µl of methanol, transferred into an HPLC auto-sampler vial and evaporated.

The aqueous residue was acidified with 100 to 200 µl of glacial acid to pH 5 and applied to a conditioned C18 solid-phase extraction (SPE) cartridge. After washing, the column was dried and stored in a vacuum desiccator over night. Elution was performed with ethyl acetate/MeOH and after adding ethyl acetate/sulfuric acid the sample was incubated for 60 min at 50 °C. Then 0.5 ml of methanolic sodium hydroxide was added and the sample was evaporated to dryness, re-dissolved in water and extracted twice with 4 ml of TBME to yield the formerly sulfated steroids. Half a millilitre of the organic layer was transferred to enable the determination of steroid concentrations,[8] the remaining volume was evaporated, re-dissolved in 2 times 100 µl of methanol, transferred into an HPLC auto-sampler vial and dried.

HPLC clean-up

Glucuronidated steroids

In order to remove all interfering or co-eluting compounds prior to GC/TC/IRMS measurements, two consecutive HPLC fractionation steps were employed. Both were performed on an Agilent 1100 HPLC system with a XBridge™ Shield RP18 5 mm (4.6 x 250 mm) column protected with a XBridge™ Shield RP18 5 mm (4.6 x 20 mm) guard column purchased from Waters (Baden-Dättwil, Switzerland). The injection volume was 50 µl and the flow rate 1 ml/min. A linear gradient was used increasing from 40/60 acetonitrile/water to 60% acetonitrile in 18 min and then within 1 min to 98% acetonitrile. After 11 min at 98%, the column was re-equilibrated for 10 min.

Before each batch of samples, a standard solution containing approximately 40 µg/ml of TESTO, EPIT, DHEA and 16EN, 200 µg/ml of PD and 100 µg/ml of 11KETO, ETIO and ANDRO each was injected twice to determine the retention times for fraction collection. Both peak shape and retention times were monitored using UV detection at a wavelength of 195 nm. The automatic fraction collector Agilent 1200 was programmed to prepare seven fractions as listed in Table 1. The different fractions were collected in small test tubes and evaporated to dryness under a stream of air. All fractions were acetylated due to considerably improved separation and peak shape of steroids on both the HPLC and the GC column.

Therefore, 50 µl of pyridine and 50 µl of acetic anhydride were added. The mixture was incubated for 60 min at 70 °C and evaporated to dryness under a stream of air and the dried residue was transferred to either GC or LC auto-sampler vials.

Fractions II (containing TESTO_Ac), III (EPIT_Ac, DHEA_Ac and 5bDIOL_Ac) and IV (ETIO_Ac, 5aDIOL_Ac) were further purified by an additional HPLC fractionation. During the second step the gradient was changed to start with 60/40 acetonitrile/water, increased to 98% acetonitrile in 16 min, was hold for 9 min and subsequent the column was re-equilibrated for 10 min at 60/40 acetonitrile/water. Again, the mixture containing acetonitrile/water was used as solvent for injection with 50 µl. A standard solution containing approximately 40 µg/mL of EPIT_Ac, TESTO_Ac, DHEA_Ac and 16EN_Ac (here as internal standard to control retention times), 200 µg/ml 5aDIOL_Ac and 5bDIOL_Ac and 100 µg/ml ETIO_Ac was injected twice to determine the retention times for fraction collection (Table 1).

Sulfated steroids

For the first clean-up step, the same gradient as for the underivatized steroids obtained from hydrolysis of glucuronidated conjugates was used. The standard mixture contained 40 µg/ml of DHEA, 5EN17a, 5EN17b and 16EN plus 100 µg/ml ETIO and ANDRO. As 16EN is not found in the fraction of sulfated steroids it was added at an absolute amount of 1 µg to each vial of samples to act as reference standard for retention time control. The fraction collector was programmed to prepare three fractions as listed in Table 2. After acetylation,

Table 1. List of different fractions collected during HPLC clean-up and separation of glucuronidated steroids presented together with relevant collection times

Fraction	Steroid	Time [min]	Fraction	Steroid	Time [min]
I	11KETO	8.8-10.0	**II_Ac**	TESTO_Ac	9.0-10.0
II	TESTO	11.3-12.5	**III_Ac**	EPIT_Ac	8.4-9.6
III	EPIT, DHEA, 5bDIOL	13.9-15.4		DHEA_Ac	10.1-11.6
IV	ETIO, 5aDIOL	15.4-17.4		5bDIOL_Ac	15.1-16.4
V	ANDRO	17.4-19.0	**IV_Ac**	ETIO_Ac	10.5-11.8
VI	PD	19.9-21.2		5aDIOL_Ac	15.3-16.6
VII	16EN	25.1-26.1			

Table 2. List of different fractions collected during HPLC clean-up and separation of sulfated steroids presented together with relevant collection times

Fraction	Steroid	Time [min]	Fraction	Steroid	Time [min]
I_S	DHEA, 5EN17a, 5EN17b	12.7-15.1	**I_S_Ac**	DHEA_Ac	8.7-10.1
II_S	ETIO, EPIA	15.1-17.4		5EN17a_Ac	14.2-15.6
III_S	ANDRO	17.4-19.0		5EN17b_Ac	15.6-17.0

fraction I_S (containing DHEA, 5EN17a and 5EN17b) was further purified using a slightly different gradient starting with 70/30 ACN/H_2O, increasing to 85% ACN in 20 min, then to 98% in 2 min, hold for 8 min and then re-equilibration for 10 min in order to achieve baseline separation of 5EN17a_Ac and 5EN17b_Ac. The collection times are listed in Table 2. All fractions were dried, reconstituted in two times 100 μL of MeOH, transferred to GC auto-sampler vials and dried before reconstitution.

The comprehensive sample clean-up enabled to measure each of the glucuronidated and most of the sulfated steroids separately. Only ETIO and EPIA could not be isolated from each other and therefore were injected together on GC. In some samples this procedure resulted in a peak overload for one of the steroids in order to achieve sufficient peak heights for the other.

GC/C/IRMS measurements

Instrumentation

All samples were measured on an Agilent 6890 Gas Chromatograph coupled to a Delta^{plus} XL gas isotope ratio mass spectrometer (ThermoElectron, Bremen, Germany) via a GC combustion interface (GCC III, ThermoElectron) where the reduction oven has been removed. Injections were performed in the splitless mode at 280 °C with injection volumes ranging from 2 to 4 μl of cyclohexane. The GC column was a J&W Scientific DB-17MS (length 30 m, i.d. 0.25 mm, film thickness 0.25 μm) from Agilent. For steroid measurements, the initial oven temperature of 70 °C was maintained for 2 min and increased at 30 °C/min up to 270 °C, then at 2 °C/min to 290 °C and then at 30 °C/min up to 300 °C and kept for 1 min. For CAL1 and CAL2 the initial oven temperature of 80 °C was maintained for 2 min and increased at 20 °C/min up to 220 °C, then at 5 °C/min to 250 °C and then at 20 °C/min up to 300 °C and kept for 2 min. Carrier gas was purified He (purity grade 4.9) with a constant flow of 1.4 and 1.0 mL/min, respectively. The thermal conversion furnace was operated at 1450 °C. Data was acquired using ISODAT® NT 2.0 software (ThermoElectron).

Sequence alignment

During HIR determinations, small and constant changes in measured δ^2H values over time are commonly observed and usually referred to as instrument drift. As long as these drifts are slow and small like the change of the virtual value of the hydrogen tank gas they do not constitute a problem and can be handled by adopting the reference gas value. If the drift is fast, i.e. several 10 ‰ during a sequence, it might still be possible to apply a linear drift correction to all values (as depicted in Figure 1). Such drifts are recognized with newly installed thermal conversion tubes or when tube replacements are required. As the data presented herein is intended to be used as a basis for reference limits in sports drug testing, we decided not to apply any post-measurement correction to our values but to monitor potential drifts carefully and to reject sequences which did not fulfill stability criteria. Therefore, each sequence of 24 to 36 samples was bracketed by a set of reference standard measurements. After determining the H_3^+-factor, a standard containing CAL2 and RSTD at 150 ng/ml was injected four times followed by a threefold injection of a standard containing the relevant steroids of interest within the sequence plus RSTD at the same concentration. At the end of the sequence the same standards were injected three times each. The results obtained for the standards from before and after were compared (t-test, $p < 0.05$) and if no significant drift occurred, the sequence was accepted.

Calibration of tank gas

The apparent δ^2H value of the hydrogen tank gas (-337 ‰) was calculated using the mean value of all 5 n-alkanes comprised in CAL1. The ascertained value for the tank gas was affirmed using CAL2 and monitored constantly as CAL2 was injected with each

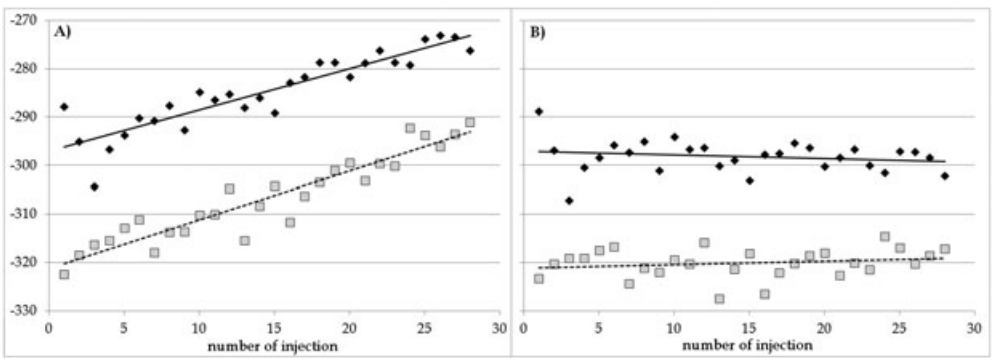

Figure 1. HIR values of CAL2 (black diamonds) and RSTD (grey squares) during a sequence of 28 measurements. A) shows the uncorrected raw data, B) the same subset of date after linear drift correction. The lines represent the linear fit. All values are in δ^2H_{VSMOW} [‰].

batch of samples. Regarding the principle of identical treatment of standard and analytes,[24] CAL2 was the only commercially available steroid reference material.

Instrument tests

Several factors, which supposedly influence the determination of HIR, were investigated prior to the reference population study. This included the impact of changes in column flow, the use of different software implemented background corrections and the 'linearity' of the instrument, i.e. the necessary amount of steroid on column to achieve repeat- and reliable measurements.

Effects originating from differences in column flow rates were tested by repeated injections of CAL2 (n = 30) by modifying the constant flow of helium from 0.5 to 2.0 ml/min.

Regarding the investigations on the different background corrections, a subset of n = 28 measurements containing both CAL2 and RSTD were recalculated with four different correction strategies (individual, median mean, dynamic and time-based) maintaining other parameters such as start- or end-slope. The time-based background was 50 s long and selected in-between both peaks.

In order to probe for 'linearity', six replicate injections of a standard mixture containing RSTD, 5aDIOL, and PD at five different concentrations between 25 and 250 µg/ml were conducted and evaluated concerning significant differences in mean values and standard deviations.

Negative control urine

Five spot urine samples from different individuals who declared no use of any prohibited substance or nutritional supplement were pooled and stored frozen at -20 °C in 20 ml portions. This urine was used for both measurements regarding repeatability and stability over time. For the experiments regarding linear mixing models, a single-spot morning urine from one healthy male individual was used to perform both sample preparations. Again, the aliquot was stored at -20 °C until preparation.

Correction for the acetate moiety

All determined values were corrected for the influence of the acetate moiety as described in literature.[21,25] All δ^2H values of urinary steroids reported within this article are for the underivatized steroid.

Method validation

The developed method for the sulfated steroids was validated by means of a linear mixing model using Equation (3)[21,26,27]:

$$\delta^2H_m = \left(\delta^2H_e - \delta^2H_a\right)\frac{c_e}{c_m} + \delta^2H_a \qquad (3)$$

with c_x = corresponding concentration and δ^2H_x = corresponding δ^2H value; subscript m stands for mixture, e for endogenous and a for added standard. For steroids found in urine as glucuronide conjugates, this validation was accomplished earlier.[21]

Repeatability of the complete procedure was tested for by six replicate preparation of one negative control urine and subsequent determination of HIR values. The method's stability over time was monitored over eight months by preparing one negative control urine with each batch of samples processed for the reference population determinations.

Results and discussion

Method validation

Repeatability

The results obtained for the six replicate sample preparation of the negative control urine are listed in Table 3. The standard deviations for the complete method were found to be in general smaller than ± 5 ‰ and therefore within the expectable range for HIR measurements.[21,28] Only for EPIA and EPIT, the values were slightly higher, most probably due to the low amount of steroid found in urine. The repeatability over time (Table 4) was noticed to be somewhat larger but still satisfactory. Over all, the improvements in sample clean up seemed to result in a superior repeatability of the method compared to the first measurements accomplished.[21] The mean standard deviation for all repeatability measurements decreased from 8.5 ‰ to 3.9 ‰ within this study.

Table 3. Repeatability of the negative control urine for a six replicate sample preparation. Listed are the mean values and the single standard deviations. All values are in δ^2H_{VSMOW}[‰]

	steroid	mean	SD
glucuronidated	11KETO	-309.5	4.4
	PD	-250.1	1.8
	16EN	-277.8	3.1
	ANDRO	-281.9	2.6
	ETIO	-303.5	2.7
	5aDIOL	-282.0	4.1
	5bDIOL	-301.7	3.2
	DHEA	-290.4	5.1
	TESTO	-266.6	3.1
	EPIT	-256.0	7.5
sulfated	ANDRO	-276.3	3.2
	ETIO	-285.8	4.5
	EPIA	-292.9	6.8
	5EN17a	-298.9	1.1
	5EN17b	-297.5	4.6
	DHEA	-294.0	4.6

Table 4. Repeatability of the negative control urine over a time period of 8 months (n = 6). Listed are the mean values and the single standard deviations. All values are in δ^2H_{VSMOV}[‰]

	steroid	mean	SD
glucuronidated	11KETO	-309.5	7.3
	PD	-253.8	6.0
	16EN	-276.5	5.9
	ANDRO	-283.9	6.5
	ETIO	-298.9	4.2
	5aDIOL	-277.9	3.6
	5bDIOL	-293.9	3.6
	DHEA	-292.3	6.5
	TESTO	-269.8	7.8
	EPIT	-261.4	6.9
sulfated	ANDRO	-277.0	1.1
	ETIO	-286.9	8.1
	EPIA	-285.6	7.6
	5EN17a	-298.3	3.5
	5EN17b	-299.1	7.8
	DHEA	-289.1	2.4

Linear mixing models

In contrast to the already published method for glucuronidated steroids, the extension to sulfated steroids needed to be validated by means of linear mixing models to ensure that the crucial step in this sample preparation – the cleavage of the sulfate moiety under acidic conditions – does not influence the HIR by any isotopic fractionation. This is of particular importance as the hydrolysis is usually not completed to 100 % yield.[8,29,30] For CIR measurements no significant influence was detected and the same was found for HIR as can be seen in Table 5. The observed standard deviations were comparable to those found with glucuronidated steroids and only for EPIA was a minor offset between the urinary values and the added standard visible. Most probably, this was due to inevitable co-elutions of the higher concentrated ETIO with the following peak of EPIA. Overall, the deconjugation from the sulfate moiety did not appear to be accompanied by any isotopic fractionation neither for carbon nor hydrogen.

Instrument tests

Influence of GC flow

The helium flow within the GC column has a direct impact on the residence time of analytes passing through the thermal conversion tubing. As the heated zone is only a short part of the complete oven pipe, influence on the completeness of thermal conversion was expected and investigated to exclude systematic errors caused by this effect. The results depicted in Figure 2 show the expected influence. Fortunately, within the normal operating range from 1.0 to 1.5 ml/min, no significant difference in measured δ^2H values was detectable. As the androstane CAL2 is chemically very similar to the investigated steroids, it can be assumed that with a stable helium flow in the designated interval, unbiased values for all measured steroids will result.

Table 5. Evaluated values for the linear mixing models referring to the least square method using equation $y = \mathbf{a}x + \mathbf{b}$. The intercept **b** represents the δ^2H-value of the added standard, the slope **a** the Δ-value between the endogenous steroid and the added standard. For comparison reasons the HIR of the added standards are also listed. SE stands for standard error, SD for standard deviation, (n = 4). All values given in δ^2H_{VSMOV} [‰]

steroid	a	SE	b	SE	Std	SD
ANDRO	35.1	3.9	-317.6	2.1	-316.2	3.0
ETIO	-61.1	3.7	-209.1	2.2	-210.3	7.4
EPIA	46.8	4.0	-328.7	2.2	-324.1	1.2
5EN17a	30.2	3.3	-322.6	1.5	-322.0	5.6
5EN17b	31.0	4.9	-322.5	2.4	-323.0	5.2
DHEA	42.8	2.4	-315.3	1.2	-314.3	5.9

Background correction

Taking into account first experiences made with HIR determinations of steroids, the influence of the employed software-implemented background correction was investigated to estimate possible systematic offsets depending on the chosen correction algorithm. And indeed, small and partly significant differences were found by recalculating the same subset of samples as listed in Table 6.

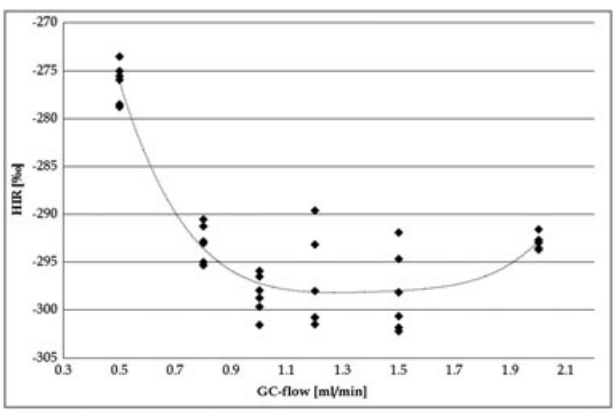

Figure 2. Influence of different constant GC flows on measured HIR of CAL2 (nominal value -297.4 ± 2.2 ‰; Indiana University). The dashed line represents a polynomial fit which only serves to assist in interpretation and not as a theoretical interpolation. All values are in δ^2H_{VSMOW} [‰].

Table 6. Mean values and standard deviations for a sequence of n = 28 measurements of a mixture containing both steroids at 150 ng/µL. All values are in δ^2H_{VSMOV} [‰]. Further information in the text

	CAL2		RSTD	
background	mean	SD	mean	SD
individual	-296.1		-318.0	
		3.39		2.96
median mean	-297.3		-323.4	
		3.21		5.62
dynamic	-296.9		-317.3	
		3.60		4.70
time based	-299.3		-323.2	
		3.65		5.35

Regarding CAL2, only the difference between the mean values of individual background in comparison to the time-based correction was found to be significant (t-test, $p < 0.01$ after Bonferroni correction). The same was found for RSTD, whereas here the mean value for dynamic background correction equals the one for the individual one. Interestingly, not only the mean value changed but also the variance is significant lower for the individual background compared to all others (F-test, $p < 0.05$). The reason for this influence created only by the software might be found in the elevated background for HIR determinations. During the measurements the background was stable at 200 and 23 mV for *m/z* 2 and 3, respectively. However, for all determinations within the study for reference-based values the individual background was chosen as far as possible.

Sample amount

Despite the fact that the ion source linearity is not an issue for HIR measurements as the suitable H_3^+ correction is software-implemented, the absolute amount of steroid necessary on-column for valid and repeatable measurement is. So the lower limit of detection regarding the used IRMS was tested by repeated injections of standards at different concentrations. The results depicted in Figure 3 show a suitable working range for the used machine set-up between 50 and 500 ng. None of the different concentration levels showed a significant difference in neither the mean values nor the variability of measurements (t-test and F-test, $p < 0.05$). For higher steroid amounts injected on column the measured HIR did not show significant differences either but the peak shape became more and more distorted by severe fronting. Lower sample sizes showed generally a drift to more enriched values together with a higher variability, whereas the latter was detected for CIR measurements, too.[7,31]

These results suggest considering urinary steroids with a concentration of at least 30 ng/ml for a 20 ml specimen, as otherwise reliable values might not be obtained due to low signal heights. Of course, this can only be defined for the herein specified setup and with a different IRMS the sensitivity can be either better or worse.

Reference-population-based values

Absolute δ^2H_{VSMOW}-values

Overall the determined HIR values ranged from -220 to -320 ‰ as can be seen in Figure 4. For a single steroid, the range is smaller and covers circa 50 ‰. In agreement with already reported CIR values,[6,8,13–15] urinary steroids show significantly different mean values in their HIR. This has already been suggested by the first results obtained on HIR and were also demonstrated within an appropriate reference population.

In the group of ERC, PD showed the most enriched value with a mean of -253.2 ‰ and 11KETO the most depleted with -296.8 ‰ while 16EN falls in between with -275.8 ‰. So the chosen ERC within this study cover the complete range of HIR found in the reference population.

For TC excreted glucuronidated, EPIT exhibited values reflecting the highest deuterium content with -257.7 ‰. Then a group containing T and the 5α-steroids 5aDIOL and ANDRO with nearly similar HIR (-273.2 ‰; -274.7 ‰ and -281.3 ‰) seems to be separated from the group encompassing DHEA, 5bDIOL and ETIO (-290.6 ‰; -292.0 ‰ and -296.1 ‰). These differences in HIR between the TC might originate from differences in their stereochemistry or the underlying metabolic pathway.[6,21,32] The impact of these findings on both doping control and studies on metabolic pathways will be discussed in an upcoming paper taking also the CIR of the investigated reference population into account.

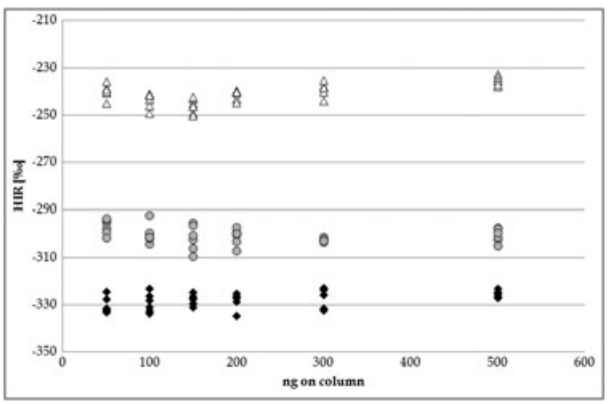

Figure 3. Influence of injected sample amount on HIR. Open triangles stand for PD, grey circles for 5aDIOL and black diamonds for RSTD. All values are in δ^2H_{VSMOW} [‰].

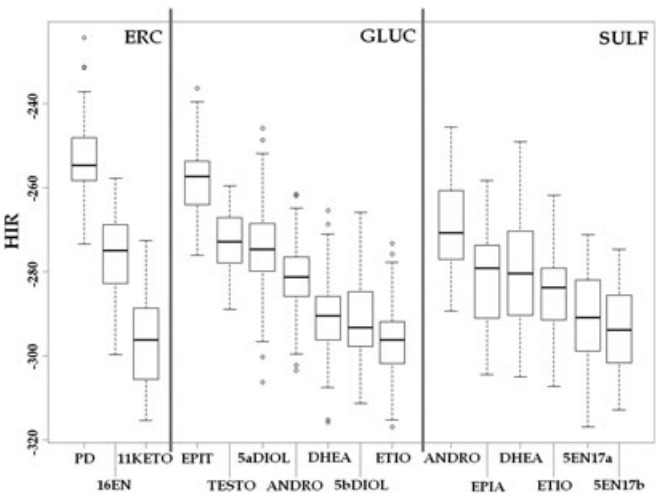

Figure 4. Boxplot of absolute HIR values found in the investigated reference population consisting of n = 67 males and females. All values are in δ^2H_{VSMOW} [‰].

For steroids excreted sulfated, ANDRO showed the most enriched mean value (-269.0 ‰), interestingly significantly different from its 3β-epimer EPIA (-281.7 ‰), which exhibited more or less similar values to DHEA and ETIO (-279.8 ‰ and -284.6 ‰). The both androstenediols 5EN17a and 5EN17b showed depleted values with -290.4 ‰ and -293.7 ‰.

As depicted circumstantially in Figure 5, steroids excreted sulfated are more enriched in deuterium than their glucuronidated counterparts. For all three steroids investigated as both excretion forms (ANDRO, ETIO and DHEA) the difference was found to be highly significant ($p < 0.001$, t-test) with a stable offset of approx. 12 ‰ between the sulfated and the glucuronidated steroid. Similar behavior was already reported for CIR and recently, different isotopic ratios at different production sites within the body were suggested as a possible explanation for this finding.[32–34] These new results corroborate the proposed explanation.

Δ-values and reference limits

In the context of doping control analysis, the differences between an ERC and an appropriate TC proved to be the most reliable and sensitive marker to detect misuse of steroids.[14] Regarding the data listed in Table 7, a similar approach will also be promising for HIR.

As outlined in Figure 4, the calculated differences for the variety of ERC spread over a large range in parallel to the absolute HIR of these steroids. The mean values calculated against the TC ANDRO for example are +28.1 ‰ if PD is used as ERC and -16.2 ‰ for 11KETO. This difference in mean values is directly reflected by the calculated lower reference limits with -0.5 ‰ (PD-ANDRO) and -55.5 for 11KETO-ANDRO. Obviously, it will not be possible to define one threshold for all pairs of ERC-TC with HIR measurements. Similar differences were also found for Δ-values calculated with CIR but were not as pronounced as for HIR.[14] Consequently,

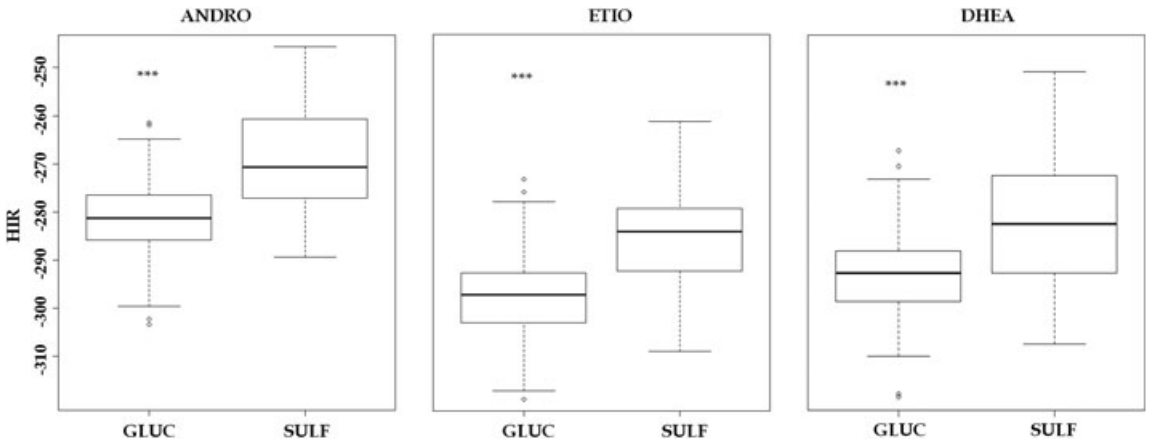

Figure 5. Comparison of steroids excreted glucuronidated and sulfated. All values are in δ^2H_{VSMOW} [‰].*** - difference was found highly significant (t-test, $p < 0.001$).

Table 7. Δ values for steroids excreted glucuronidated within the investigated reference population encompassing n = 67 males and females. Listed are the number of accomplished measurements (n), the mean values, the standard deviations and the resulting reference limits obtained by adding (RL up) or subtracting (RL down) the threefold standard deviation. All values given in δ^2H_{VSMOV} [‰]

	PD-ANDRO	PD-ETIO	PD-TESTO	PD-EPIT	PD-DHEA	PD-5aDIOL	PD-5bDIOL
n	67	67	31	39	55	54	57
mean	28.1	42.9	21.9	4.4	36.6	21.2	38.1
SD	9.67	9.76	13.04	9.61	11.81	14.66	11.79
RL up	57.2	72.2	61.0	33.2	72.0	65.2	73.5
RL down	-0.9	13.6	-17.2	-24.4	1.1	-22.8	2.7
	16EN-ANDRO	**16EN-ETIO**	**16EN-TESTO**	**16EN-EPIT**	**16EN-DHEA**	**16EN-5aDIOL**	**16EN-5bDIOL**
n	62	62	31	39	52	52	54
mean	6.4	21.3	-1.8	-16.3	14.6	0.0	16.6
SD	10.75	10.09	11.77	13.13	11.34	14.33	12.22
RL up	38.6	51.6	33.5	23.1	48.7	43.0	53.2
RL down	-25.8	-9.0	-37.1	-55.7	-19.4	-43.0	-20.1
	11KETO-ANDRO	**11KETO-ETIO**	**11KETO-TESTO**	**11KETO-EPIT**	**11KETO-DHEA**	**11KETO-5aDIOL**	**11KETO-5bDIOL**
n	62	62	29	37	52	51	53
mean	-16.2	-0.7	-24.3	-39.6	-7.5	-24.4	-5.9
SD	13.11	12.22	12.10	12.34	15.41	15.15	13.31
RL up	23.2	36.0	11.9	-2.6	38.7	21.1	34.0
RL down	-55.5	-37.3	-60.6	-76.6	-53.7	-69.8	-45.9

reference-population-based decision limits appear to be the only possible way to use HIR in sports drug testing. Another benefit of these limits is their outstanding robustness.[35] All samples used to calculate the thresholds have been processed in the same way, and doping control samples will undergo the identical treatment. Any systematical offset is already implemented in these decision limits. The same holds true for the measurement uncertainty, which is directly factored in the calculation. The use of reference based values is highly recommended by the International Federation of Clinical Chemistry and Laboratory Medicine.[36]

In principle, the calculated limits will be valid on both sides of the distribution resulting in an upper and lower limit as listed in Table 7. The results so far suggest that the lower limit will be of greater importance for doping control as the majority of testosterone preparations investigated so far showed less depleted values than endogenous steroids.[17] This is also supported by the results obtained for the excretion study which will be presented later on. But as there is only one study dealing with the HIR of steroid preparations published until now, the presence of artificial steroid exhibiting more depleted values than endogenous steroids cannot be excluded. Therefore, at least the theoretical occurrence of values beyond the upper reference limit has to be considered and the values are listed in Table 7, too.

As the reliability of reference based thresholds increases with the number of subjects investigated, the quantity of accomplished measurements for each pair of Δ-values has been added. Obviously, especially for TESTO and EPIT only about one half of the investigated specimens could be completed to yield reliable data. This was mainly due to low concentrations of these steroids in female samples and accordingly the resulting reference values might not be as trustworthy as the ones for all other steroids.

In Table 8, the results for Δ-values calculated from steroids excreted sulfated into urine are exemplarily listed with PD as ERC. In principle, the results are similar. The main drawback here was the lacking of an ERC excreted sulfated. In a former study on CIR, pregn-5-ene-3β,17α,20α-triol was employed.[8] Unfortunately, urinary concentrations of this steroid turned out to be too low for reliable HIR measurements. In the case of a testosterone administration, all steroids with 5-ene-conformation could serve as ERC as they proved not to be influenced by a TESTO administration (unpublished data).

Factors influencing HIR

Several factors influencing the CIR of endogenous steroids have been identified.[13–15,32] For HIR, no significant influence of gender or exercise could be found. In parallel to CIR, the excreted steroid concentration was not correlated with isotopic ratios in any way. But significant differences between glucuronidated and sulfated steroids were found for both carbon and hydrogen (*vide supra*).

Excretion study

An excretion study with 100 mg of TESTO-undecanoate administered orally was conducted to assess the utility of the established reference thresholds and their applicability in doping controls. According to Figure 6, both major metabolites of TESTO, ANDRO and ETIO were found significantly influenced directly after steroid administration while the ERC PD remained stable over the complete study period. The Δ-values exceeded the reference-based thresholds for 9 and 18 h after ingestion for ANDRO and ETIO, respectively. This detection window was found comparable to the one for CIR within this study (data not shown). The influence on the 5β-steroid ETIO was more pronounced than on the 5α-steroid ANDRO. Similar results were obtained for both androstanediols, where 5bDIOL showed a stronger influence than 5aDIOL, which was not elevated beyond the established threshold (Figure 7). TESTO itself was influenced beyond the reference-based limit only for a short time period (*ca.* 6 h).

Interestingly, the urinary excreted TESTO did not reach the δ^2H_{VSMOW}-value of the administered steroid. While the excreted TESTO showed values around -250 ‰, the HIR of the administered steroid was found to be -208 ‰ (n = 24, standard deviation ± 4.7 ‰). Simultaneous CIR measurements on these samples yielded a different picture. Here the $\delta^{13}C_{VPDB}$-value of the administered steroid was perfectly reflected by the excreted TESTO (-27.0 and -27.1 ‰, respectively). This is in agreement with other excretion studies reporting that directly after oral administration of a steroid it is excreted into urine in very high amounts, entirely covering/masking the endogenous counterpart.[6,32] At the moment there is no coherent explanation for the observed

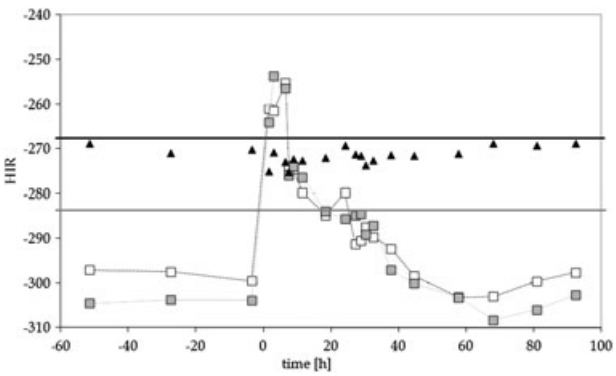

Figure 6. Changes of HIR over the course of time after oral administration of 100 mg TESTO- undecanoate at t = 0h. Black triangles represent PD, open squares ANDRO and grey squares ETIO. The solid black line demonstrates the calculated reference limit for PD-ANDRO, the grey line the one for PD-ETIO. All values are in δ^2H_{VSMOW} [‰].

Table 8. Δ values for target steroids excreted sulfated within the investigated reference population encompassing n = 67 males and females. Listed are the number of accomplished measurements (n), the mean values, the standard deviations and the resulting reference limits obtained by adding (RL up) or subtracting (RL down) the threefold standard deviation. All values given in δ^2H_{VSMOV} [‰]

	PD-ANDRO	PD-ETIO	PD-EPIA	PD-DHEA	PD-5EN17a	PD-5EN17b
n	56	56	55	54	55	57
mean	15.7	31.0	28.9	26.1	36.4	40.0
SD	11.13	10.82	11.89	12.90	12.05	9.85
RL up	49.1	63.4	64.6	64.8	72.5	69.6
RL down	-17.7	-1.5	-6.7	-12.6	0.2	10.5

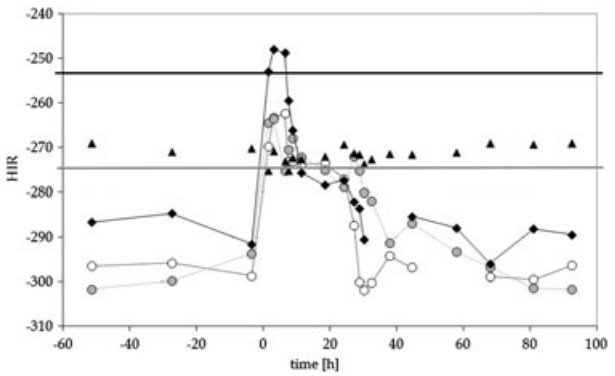

Figure 7. Changes of HIR over the course of time after oral administration of 100 mg TESTO- undecanoate at t = 0h. Black triangles represent PD, open circles 5aDIOL, grey circles 5bDIOL and black diamonds TESTO. The solid black line demonstrates the calculated reference limit for PD-TESTO, the grey line the one for PD-5bDIOL. All values are in δ^2H_{VSMOW} [‰].

differences. Maybe hydrogen in the steroid backbone can exchange with body water very rapidly or during cleavage of the TESTO-undecanoate isotopic fractionation is taking place for HIR. Further studies to clarify this topic are ongoing.

Overall, the excretion study demonstrated the usefulness of HIR in the context of sports drug testing. The method enables unambiguous separation of post-administration from negative control urine samples in a very similar way to the well-established CIR.

Application to a real sample

Several years ago, an interesting sample was investigated in the Norwegian Doping Control Laboratory in Oslo. The sample exhibited a strongly increased TESTO/EPIT ratio of approx. 100 due to a very high urinary TESTO concentration of more than 500 ng/ml. These values were confirmed and the sample did not show any signs of degradation or the like. So it was forwarded to IRMS and the obtained results are listed in Table 9. In comparison with a positive quality control (QC_Pos) urine obtained by pooling urines of a TESTO excretion study and a negative QC (QC_Neg), pooled pre-administration urines from the same subject, the suspicious sample yielded completely negative CIR values and all differences were found well in-between the respective reference intervals.

The method presented in here, especially the defined reference-based thresholds, enabled reanalysis of the suspicious sample and to test for the possibility if the athlete did misuse a TESTO preparation with a similar CIR value to endogenous steroids. As only a remainder of the B-sample was left, this investigation was only performed for scientific reasons. The results are listed in Table 10. Obviously, all the target analytes under investigation were found influenced. For TESTO itself, for ANDRO, ETIO and 5bDIOL the measured Δ-values fall below the established thresholds. This does indicate with a probability of >99.8% for each Δ-value that the found steroidal HIR in the suspicious urine specimen are not in accordance with normally occurring values of endogenous steroids.

Again, the presented method proved its possible merit for doping control analysis. In contrast to CIR measurements, HIR could clearly identify the exogenous origin of the urinary TESTO and its metabolites.

To what extent it will be possible to combine HIR and CIR measurements in the context of doping control analysis to improve the detection power of the isotope method in a whole will be investigated in the future. So far it can be stated that HIR give complementary information and can be used in parallel to CIR.

Table 9. $\Delta^{13}C$ values for the Norwegian sample together listed with a positive and a negative quality control sample. All values given in $\delta^{13}C_{VPDB}$ [‰]

CIR	PD-A	PD-E	PD-5a	PD-5b	PD-T
QC_Pos	4.0	4.5	4.5	4.2	5.6
QC_Neg	0.6	1.6	1.3	2.1	1.8
sample	0.7	2.1	1.7	1.7	2.3

Conclusion

The already-existing method to determine HIR of urinary steroids has been improved, extended to steroids excreted sulfated into urine and the first comprehensive reference population has been investigated to elucidate normal values and to enable calculation of reference based thresholds for doping control analysis. The validity of the method as a whole and of the measurements in particular was carefully investigated to ensure reliable data was obtained. The usefulness of the new reference limits was tested for by an excretion study with TESTO undecanoate and the influence of the administered steroid on the HIR of endogenous urinary steroids was clearly visible. Most of the target analytes were found elevated beyond the established thresholds in a similar way to CIR. These findings impressively demonstrate the potential and value HIR can posses in the field of sports drug testing.

One sample with an abnormally high TESTO/EPIT ratio and negative CIR found positive with the HIR method supports the hypothesis that recent concerns over increasing reports of TESTO preparation on the black market with 'endogenous' carbon signatures can be addressed by the presented method. Here, further investigations will be necessary as the urinary steroids did not completely reflect the HIR of the administered one as is known for CIR. Possible exchange of hydrogen with body water or the influence of drinking water over all will be subject to another oncoming investigation.

Furthermore, the potential of combining both isotope ratios of hydrogen and carbon to increase the sensitivity of IRMS in doping control will have to be investigated in the near future.

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Table 10. Δ^2H values for the Norwegian sample listed together with a positive and a negative quality control sample. The available reference limits (RL down) are also presented for comparison reasons. All values given in δ^2H_{VSMOV} [‰]

HIR	PD-A	PD-E	PD-5a	PD-5b	PD-T
QC_Pos	-23.3	-8.6	-18.7	-14.3	-35.6
QC_Neg	19.6	44.4	19	35.2	28.5
sample	**-8.6**	**8.3**	**-4.9**	**1.7**	**-24.3**
RL down	-0.9	13.6	-22.8	2.7	-17.2

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References

[1] C. Saudan, N. Baume, P. Mangin, M. Saugy. Urinary analysis of 16(5α)-androsten-3α-ol by gas chromatography/combustion/isotope ratio mass spectrometry: Implications in anti-doping analysis. *J. Chromatogr. B* **2004**, *810*, 157.

[2] C. Saudan, A. Desmarchelier, P.E. Sottas, P. Mangin, M. Saugy. Urinary marker of oral pregnenolone administration. *Steroids* **2005**, *70*, 179.

[3] M. Hebestreit, U. Flenker, G. Fußhöller, H. Geyer, U. Güntner, U. Mareck, T. Piper, M. Thevis, C. Ayotte, W. Schänzer. Determination of the origin of urinary norandrosterone traces by gas chromatography combustion isotope ratio mass spectrometry. *Analyst* **2006**, *131*, 1021.

[4] A.T. Cawley, G.J. Trout, R. Kazlauskas, A.V. George. The detection of androstenedione abuse in sport: a mass spectrometry strategy to identify the 4-hydroxyandrostenedione metabolite. *Rapid Commun. Mass Spectrom.* **2008**, *22*, 4147.

[5] C. Buisson, C. Mongongu, C. Frelat, M. Jean-Baptiste, J. de Ceaurriz. Isotope ratio mass spectrometry analysis of the oxidation products of the main and minor metabolites of hydrocortisone and cortisone for antidoping controls. *Steroids* **2009**, *74*, 393.

[6] T. Piper, P. Riemann, G. Opfermann, U. Mareck, H. Geyer, G. Vajiala, U. Flenker, W. Schänzer. Determination of $^{13}C/^{12}C$ ratios of urinary epitestosterone and its main metabolites 5α- and 5β-androstane-3α,17α-diol. *Drug Test. Anal.* **2009**, *1*, 576.

[7] T. Piper, H. Geyer, V. Gougoulidis, U. Flenker, W. Schänzer. Determination of $^{13}C/^{12}C$ ratios of urinary excreted boldenone and its main metabolite 5β-androst-1-en-17β-ol-3-one. *Drug Test. Anal.* **2010**, *2*, 217.

[8] T. Piper, G. Opfermann, M. Thevis, W. Schänzer. Determination of $^{13}C/^{12}C$ ratios of endogenous urinary steroids excreted as sulpho conjugates. *Rapid Commun. Mass Spectrom.* **2010**, *24*, 3171.

[9] A.T. Cawley, G.J. Trout, R. Kazlauskas, A.V. George. The potential of urinary androstdiene markers to identify 4-androstendiol (4-ADIOL) administration in athletes. *Forensic Sci. Int.* **2011**, *208*, 129.

[10] T. Piper, C. Schlug, U. Mareck, W. Schänzer. Investigations on changes in $^{13}C/^{12}C$ ratios of endogenous urinary steroids after pregnenolone administration. *Drug Test. Anal.* **2011**, *3*, 283.

[11] T. Piper, G. Fusshöller, C. Emery, W. Schänzer, M. Saugy. Investigations on carbon isotope ratios and concentrations of urinary formestane. *Drug Test. Anal.* **2012**, DOI: 10.1002/dta.386

[12] M. Ueki, M. Okano. Analysis of Exogenous Dehydroepiandrosterone Excretion in Urine by Gas Chromatography/Combustion/Isotope Ratio Mass Spectrometry. *Rapid Commun. Mass Spectrom.* **1999**, *13*, 2237.

[13] U. Flenker, U. Güntner, W. Schänzer. $\delta^{13}C$-values of endogenous urinary steroids. *Steroids* **2008**, *73*, 408.

[14] T. Piper, U. Mareck, H. Geyer, U. Flenker, M. Thevis, P. Platen, W. Schänzer. Determination of $^{13}C/^{12}C$ ratios of endogenous urinary steroids: method validation, reference population and application to doping control purposes. *Rapid Commun. Mass Spectrom.* **2008**, *22*, 2161.

[15] A.T. Cawley, G.J. Trout, R. Kazlauskas, C.J. Howe, A.V. George. Carbon isotope ratio ($\delta^{13}C$) values of urinary steroids for doping control in sport. *Steroids* **2009**, *74*, 379.

[16] T. Piper, U. Flenker, U. Mareck, W. Schänzer. $^{13}C/^{12}C$ ratios of endogenous urinary steroids investigated for doping control purposes. *Drug Test. Anal.* **2009**, *1*, 65-72.

[17] A.T. Cawley, M. Collins, R. Kazlauskas, D.J. Handelsman, R. Heywood, M. Longworth, A. Arenas-Queralt. Stable isotope ratio profiling of testosterone preparations. *Drug Test. Anal.* **2010**, *2*, 557.

[18] G. Forsdahl, C. Östreicher, M. Koller, G. Gmeiner. Carbon isotope ratio determination and investigation of seized testosterone preparations. *Drug Test. Anal.* **2011**, *3*, 814.

[19] A.L. Sessions, T.W. Burgoyne, A. Schimmelmann, J.M. Hayes. Fractionation of hydrogen isotopes in lipid biosynthesis. *Organic Geochem.* **1999**, *30*, 1193.

[20] U. Güntner, U. Flenker, C. Ayotte, M. Ueki, W. Schänzer. *Recent Advances in Doping Analysis (12)*, (Eds: W. Schänzer, H. Geyer, A. Gotzmann, U. Mareck), Sport&Buch Strauß: Cologne, **2004**, pp. 251.

[21] T. Piper, M. Thevis, U. Flenker, W. Schänzer. Determination of the deuterium/hydrogen ratio of endogenous urinary steroids for doping control purposes. *Rapid Commun. Mass Spectrom.* **2009**, *23*, 1917.

[22] T.B. Coplen. Guidelines and recommended terms of expression of stable-isotope-ratio and gas-ratio measurement results. *Rapid Commun. Mass Spectrom.* **2011**, *25*, 2538.

[23] U. Mareck, H. Geyer, G. Opfermann, M. Thevis, W. Schänzer. Factors influencing the steroid profile in doping control analysis. *J. Mass Spectrom.* **2008**, *43*, 877.

[24] R.A. Werner, W.A. Brand. Referencing Stategies and Techniques in Stable Isotope Ratio Analysis. *Rapid Commun. Mass Spectrom.* **2001**, *15*, 501.

[25] G. Docherty, V. Jones, R.P. Evershed. Practical and theoretical considerations in the gas chromatography/combustion/isotope ratio mass spectrometry $\delta^{13}C$ analysis of small polyfunctional compounds. *Rapid Commun. Mass Spectrom.* **2001**, *15*, 730.

[26] C.D. Keeling. The concentration and isotopic abundance of atmospheric carbon dioxide in rural areas. *Geochim. Cosmochim. Acta* **1958**, *13*, 322.

[27] D.E. Pataki, J.R. Ehlering, L.B. Flanagan, D. Yakir, D.R. Bowling, C.J. Still, N. Buchmann, J.O. Kaplan, J.A. Berry. The application and interpretation of Keeling plots in terrestrial carbon cycle research. *Global Biogeochem. Cycles* **2003**, *17*, 1022.

[28] A.L. Sessions. Isotope-ratio detection for gas chromatography. *J. Sep. Sci.* **2006**, *29*, 1946.

[29] N.B. Talbot, J. Ryan, J.K. Wolfe. The Determination of Sodium Dehydroisoandrosterone Sulfate in Water or Urine. *J. Biol. Chem.* **1943**, *148*, 593.

[30] S. Burstein, S. Lieberman. Hydrolysis of Ketosteroid Hydrogen Sulfates by Solvolysis Procedures. *J. Biol. Chem.* **1958**, *233*, 331.

[31] D.A. Merritt, J.M. Hayes. Factors Controlling Precision and Accuracy in Isotope-Ratio-Monitoring Mass Spectrometry. *Anal. Chem.* **1994**, *66*, 2336.

[32] T. Piper, C. Emery, M. Saugy. Recent developments in the use of isotope ratio mass spectrometry in sports drug testing. *Anal. Bioanal. Chem.* **2011**, *401*, 433.

[33] T. Piper, H. Geyer, W. Schänzer. Degradation of urine samples and its influence on the $^{13}C/^{12}C$ ratios of excreted steroids. *Drug Test. Anal.* **2010**, *2*, 620.

[34] T. Piper, N. Baume, E. Strahm, C. Emery, M. Saugy. Influences of β-HCG administration on carbon isotope ratios of endogenous urinary steroids. *Steroids* **2012**, *77*, 644.

[35] H.E. Solberg. The IFCC recommendation on estimation of reference intervals. The RefVal Program. *Clin. Chem. Lab. Med.* **2004**, *42*, 710 (and references therein).

[36] H.E. Solberg. A guide to IFCC Recommendations on Reference Values. *J. Int. Fed. Clin. Chem.* **1993**, *5*, 160.